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IL-10 und TGF-ß1 Plasmaspiegel bei atopischen Hunden vor und während der Immunotherapie

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Zusammenfassung (Deutsch)

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IL-10 und TGF-ß1 Plasmaspiegel bei atopischen Hunden vor und während der Immunotherapie

Studien aus der Humanmedizin deuten darauf hin, dass die Zytokine IL-10 und TGF-ß1 bei der allergenspezifischen Immuntherapie (ASIT) eine wichtige Rolle spielen könnten. Wenig ist bekannt über die Funktion dieser Zytokine bei atopischen Hunden.
Dieser Studie verglich die IL-10 und TGF-ß1 Plasmaspiegel bei atopischen Hunden und Kontrollhunden und untersuchte ihr Verlauf während vier ASIT: intralymphatisch (ILIT), subkutan (SCIT), sublingual (SLIT) und rekombinant Derf 2 (Allermmune®).
54 atopische Hunde und 32 Kontrollhunde wurden eingeschlossen. Bei 30 atopischen Hunden wurde eine ASIT durchgeführt.
Die Hunde, die mit einer ASIT behandelt wurden, wurden in 4 Gruppen eingeteilt: ILIT n = 10, SCIT n = 5, SLIT n = 4 und Allermmune® n = 11. Die Blutproben wurden nach 0, 3, 6 und 12 Monaten in der ILIT-, SCIT- und SLIT-Gruppe und nach 0, 1.5 und 3 Monaten in der Allermmune®-Gruppe entnommen. Pruritus Score (PVAS), Canine Atopic Dermatitis Severity Index (CADESI-4) und verabreichte Medikamente (MS) wurden zu jedem Zeitpunkt aufgezeichnet. Zur Quantifizierung von IL-10 und TGF-ß1 im Plasma wurden kommerziell erhältliche ELISA-Kits verwendet.
Es gab keinen signifikanten Unterschied zwischen atopischen und Kontrollhunden. Die IL-10-Spiegel waren in der ILIT-Gruppe am Ende der Studie signifikant erhöht.
Diese Ergebnisse deuten darauf hin, dass IL-10 und TGF-ß1 bei atopischen Hunden nicht verändert zu sein scheinen und, dass IL-10 eine Rolle beim Mechanismus der ILIT spielen könnte.

Schlüsselwörter:
1. Hund
2. Atopische Dermatitis
3. Immuntherapie
4. IL-10
5. TGF-ß1

Zusammenfassung (Englisch)

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IL-10 and TGF-ß1 plasma levels in atopic dogs before and during immunotherapy

Results of human studies suggest that the cytokines IL-10 and TGF-ß1 may play an important role in allergen-specific immunotherapy (ASIT). There is little known about the function of these cytokines in atopic dogs.
This study compared the plasma levels of IL-10 and TGF-ß1 in atopic and control dogs and investigated their changes during four immunotherapies: intralymphatic (ILIT), subcutaneous (SCIT), sublingual (SLIT) and recombinant Derf 2 (Allermmune®). A total of 54 atopic dogs and 32 control dogs were included. Immunotherapy was performed in 30 atopic dogs.
The dogs undergoing immunotherapy were allocated to 4 groups: ILIT n = 10, SCIT n = 5, SLIT n = 4 and Allermmune® n = 11. Blood samples were collected at 0, 3, 6 and 12 months in the ILIT, SCIT and SLIT group, and at 0, 1.5 and 3 months in the Allermmune® group. Canine atopic dermatitis extent and severity index (CADESI-4), pruritus visual analog scale (PVAS) and medications score (MS) were recorded at each timepoint. Commercially available ELISA kits were used to quantify IL-10 and TGF-ß1 in plasma.
There was no significant difference in IL-10 and TGF-ß1 between atopic and control dogs. The IL-10 levels were significantly increased in the ILIT group at the end of the study.
The findings of this work suggest that IL-10 and TGF-ß1 do not seem to be altered in atopic dogs and that IL-10 may play a role in the mechanism of ILIT.

Keywords:
1. Dogs
2. Atopic Dermatitis
3. Immunotherapy
4. IL-10
5. TGF-ß1

Introduction

Canine atopic dermatitis (CAD) is a chronic inflammatory and pruritic skin disease with a genetic predisposition. CAD is often associated with high-level of IgE antibodies specific to environmental allergens.[1,2] Its diagnosis relies on the patient history, clinical examination and the exclusion of other similarly presenting diseases such as food and flea allergies, ectoparasite infestations, pyoderma and *Malassezia* dermatitis.[3]
To date, allergen-specific immunotherapy (ASIT) is the only available and effective etiologic treatment for CAD, even though reported response rates vary.[4–6] Several different approaches have been proposed, namely subcutaneous (SCIT), intralymphatic (ILIT), recombinant Derf 2 (Allermmune®; ZENOAQ, Tokyo, Japan) and sublingual (SLIT) immunotherapy. These types of immunotherapy have recently been compared and the first three have been shown to be associated with better results compared to SLIT.[7–9]
Despite its proven efficacy, the exact mechanism of action of ASIT is still unclear. In human medicine, there is growing evidence that T-regulatory cells (Tregs) and the cytokines IL-10 and TGF-ß1 play an important role in this mechanism. [10–12]
In veterinary medicine, very few studies focused on Tregs, IL-10 and TGF-ß1 in CAD and their role during ASIT. Two studies recently reported significant higher Tregs percentage in atopic dogs compared to healthy control dogs.[13,14] A third study showed significant increase in Tregs values after allergen challenge.[15] ASIT has been associated with an increase in circulating Tregs, however pre-immunotherapy values did not differ from those of healthy dogs.[16]
Tregs are defined by various cell markers and several of them are still not available for dogs and comparisons between studies and/or species are also not always possible.[17–19]
The study of the production of regulatory cytokines seems also an easier and more logical approach. Two studies reported higher levels of IL-10 in control dogs compared to atopic dogs,[20,21] whereas another author showed opposite results.[22] There were no differences between the two groups were observed in another study.[23] Controversial results on TGF-ß1 are also reported, with atopic dogs showing higher,[20] lower[21,24] or similar[22,23] values as the control dogs.
The present study aims to compare the circulating protein levels of the regulatory cytokines IL-10 and TGF-ß1 in atopic and control dogs. Then to investigate their changes in atopic dogs undergoing four different options of immunotherapy, namely SCIT, SLIT, ILIT and Allermmune®.

Material and methods

The present work is part of three previous published clinical studies which analyzed the efficacy of different ASIT protocols on atopic dogs.[7–9]
This study was approved by our institution's animal care and all dogs entered the study with the owner's informed consent. We included cases presented to the veterinary hospital of the University of Zurich and beagle dogs housed in a research facility belonging to the University of Zurich as part of the control group.

Atopic dermatitis group

The diagnosis of AD was made using standard criteria.[25] Briefly, ectoparasite infections were ruled out with negative skin-scraping results and antiparasitic treatment and bacterial and yeast infection were treated before inclusion. All dogs underwent an elimination diet, followed by a challenge with the regular food to rule out food allergy. Dogs that were allergic to food were excluded. A total of 54 client-owned dogs with atopic dermatitis (AD)

were enrolled in the study: 43 were dogs included in two previous studies[8,9] while 11 additional dogs were recruited and treated with the same inclusion criteria and protocol as in another recent study.[7] Immunotherapy was performed in 30 dogs, which were bled at several time points. Those dogs were divided in four different groups: ILIT (n=10), SCIT (n=5), SLIT (n=4) and Allermmune® (n=11). The remaining 24 dogs were only used for the comparison with healthy dogs. We used an IgE-Serology assay (Heska Allercept™ System, Heska AG, Fribourg, Switzerland) and/or intradermal testing to identify any allergens involved in provoking an immune response. All the dogs in the study tested positive for at least one allergen.

Control group

The control group was composed of 32 non-allergic dogs: 25 were client-owned dogs presented to the veterinary hospital of the University of Zurich between October and December 2019 while seven were adult, intact, clinical healthy beagle dogs housed in a research facility belonging to the University of Zurich. The control group dogs were included if they had no history or clinical signs of skin diseases or conditions likely to modify the immune reactions (eg. infections, neoplasia, immune-mediated or endocrine diseases) and were not receiving systemic immunosuppressive agents.

Immunotherapy protocols

The allergens included in the desensitization solution were chosen based on the history of the dog and the allergen test results. In the Allermmune® group, all dogs were mainly sensitized to *Dermatophagoides farinae* (*D. farinae*) and received consequently only Derf 2 allergen. The route of administration of the vaccine was chosen by the owners, and the desensitization-protocols were performed according to the methods already described in the previous studies.[7,8] Briefly, SCIT was performed with subcutaneous injections following the manufacturer's protocol. In the SLIT, the allergen solution was administered orally twice daily. The ILIT treatment was given in the author's clinic and consisted of 4 monthly intralymphatic injections. After this, subcutaneous injections were performed for the rest of the study period. Allermmune® immunotherapy was also performed in the author's clinic, with 6 weekly subcutaneous injections followed by 2 monthly injections.

Assessment of clinical response

The dogs in the SCIT, SLIT or ILIT group, had clinical assessments and collection of blood samples performed at the start of the study (zero months) and, three, six, and 12 months later.

The assessment and sample collection of the dogs in the Allermmune® group was performed at study inclusion (zero months) and after one-and-a-half and three months. All included dogs Canine Atopic Dermatitis Extent and Severity Index (CADESI-04), pruritus Visual Analog Scale (pVAS) and medication scores were recorded at each examination.[26–28] The method for evaluation of medication score (MS) performed as previously described.[9] The efficacy of the interventions was assessed by comparing the scores at the start and at the end of the study. The dogs were considered to be "responders" if there was a ≥50% improvement in the CADESI-04 and pVAS score with a stable or improved MS.

Blood-samples collection and determination of IL-10 and TGF-ß1 plasma level with ELISA

Whole blood was collected by venipuncture into tubes containing ethylenediaminetetraacetic acid (EDTA) at the start of the studies and each follow-up examination. The blood was centrifuged (1580 x g, 22°C for 10 min) and the plasma was collected and stored at -80°C until further analysis. Two commercially available validated kits (Canine IL-10 Quantikine and Mouse/Rat/Porcine/Canine TGF-β1 Quantikine ELISA Kit; R&D Systems, Minneapolis, MN, USA) were used for the quantitation of IL-10 and TGF-ß1 in plasma following the manufacturer's instructions. The experimentally determined detection limits were 3.9 pg/mL for the IL-10 and 31.3 pg/mL for the TGF-β1 kit (data not shown).

Several values measured with the IL-10 ELISA Kit were below the detection limit. We decided to report those values and include them in our statistics, with a statement of their uncertainty in the discussion. This was done according to the recommendations of the Analytical Methods Committee of the Royal Society of Chemistry.[29] Additionally, we have repeated the analyses after exclusion of values under the detection limit and any conflicting results have been mentioned.

Statistical Analysis

Non-parametrical statistical tests were performed since our dataset did not follow a normal distribution Kolmogorov-Smirnov test (KS). The Mann-Whitney test was employed for the following comparison: allergic and control dogs, responders and non-responders, dogs receiving systemic medications and dogs without systemic immunomodulatory treatments. The correlation between the cytokine concentrations and the clinical scores, and between the IL-10 and the TGF-ß1 concentrations were measured with a Spearman-rank Test. The proportion of dog that had IL-10 values below the detection limit in both the allergic and the control group were compared with the Fisher's Exact Test. The Kruskal-Wallis test was used for comparison between the four immunotherapy groups over time. A *P*-value ≤ 0.05 was considered significant. The analyses were performed with the software Graphpad Prism 8.0 (Graphpad 8.0. La Jolla, CA, USA).

Results

A total of 54 atopic dogs and 32 control dogs were included in the study. The mean age of the allergic group was 4.5 years (range: 1-12 years), whereas the mean age for the control group was 5.2 years (range: 1-14 years). In the allergic group, there were 28 female and 26 male dogs, in the control group 15 females and 17 males. Various breeds were included; French Bulldogs (n = 10) and West Highland White Terriers (n = 6) were over represented in the allergic group. Beagle dogs (n = 7) are over represented in the control group. IL-10 and TGF-ß1 plasma levels in both the allergic and control group are summarized in Table 1 and Figure 1. There was no statistical difference between the two groups. Statistical analyses are not modified when IL-10 values under the detection limit are excluded. Removing one outlier with an IL-10 value of 1095.4 pg/mL did not affect these results.

Table 1. Comparison of IL-10 and TGFß-1 plasma levels in allergic and control dogs.

Group	Number of dogs	IL-10 (pg/ml)		TGF-ß1 (pg/ml)	
		Mean (± SD)	Median	Mean (± SD)	Median
Allergic dogs	54	31.09 (± 151.8)	1.828	15642 (± 15429)	7580
Control dogs	32	43.57 (± 99.03)	1.953	13814 (± 11292)	9233

S.D.: Standard Deviation

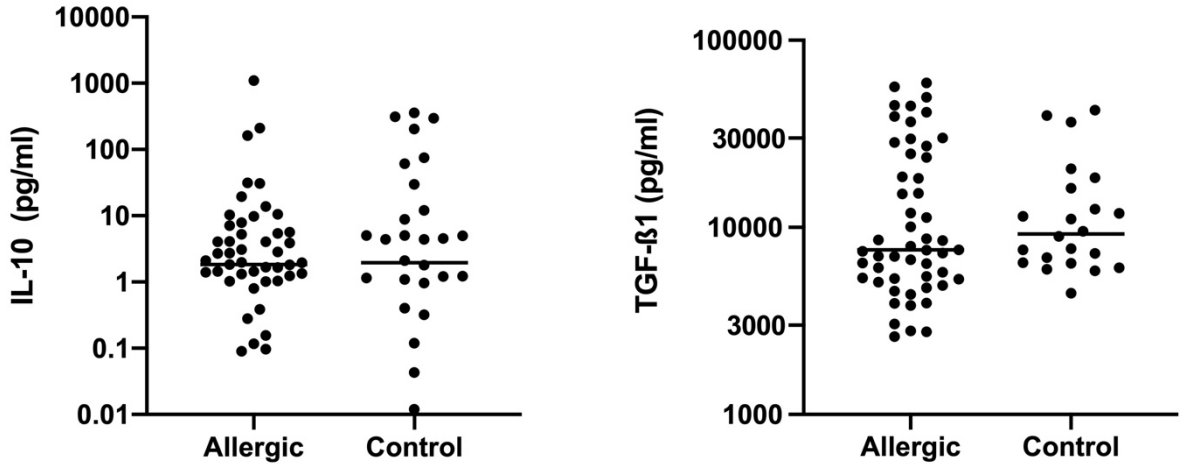

Figure 1. Comparison of IL-10 and TGF-ß1 concentrations (on a logarithmic scale) in allergic and control dogs. The horizontal line indicates the median.

The measured IL-10 concentrations of several plasma samples were below the detection limit of the essay in both the allergic- and the control group. In the allergic group, 33.3% (18/54) of the samples were above the detection limit, whereas in the control group the detectable values were 46.8% (15/32). The proportions of dogs with values within the range of the assay were not statistically different between the two groups.
No correlation between the IL-10- and TGFß-1 concentrations was detected.
In the allergic dogs' group, no correlation was found between the IL-10 and TGFß-1 plasma levels and the clinical score (CADESI) of the dogs.
The different medications of the allergic dogs are summarized in Table 2.

Table 2. Medications of the dogs in the allergic group with respective IL-10 and TGFß-1 plasma concentrations.

		IL-10 (pg/ml)		TGF-ß1 (pg/ml)	
Type of medication	**Number of dogs**	**Mean (± SD)**	**Median**	**Mean (± SD)**	**Median**
Systemic medications	32	9.259 (± 29.56)	1.754	14033 (± 15465)	7514
No systemic medications	22	63.57 (± 234.7)	1.953	17836 (± 15464)	11331

S.D.: Standard Deviation

No statistical difference in IL-10 and TGFß-1 plasma concentrations was found between dogs under systemic medications and dogs without systemic immunomodulatory medications. In the topical- or no-medications group one IL-10 value was very high (1095.4 pg/mL) compared to the others. The removal of this outlier did not influence the result of the statistical analysis.

A total of 30 dogs were followed during immunotherapy: ILIT group (n = 10), SCIT group (n = 5), SLIT group (n = 4) and Allermmune® group (n = 11). At the initiation of the immunotherapy (0 months) the groups did not differ for IL-10 and TGFß-1 plasma concentrations, CADESI, pVAS and MS.
Overall, no statistical significance was found for IL-10 and TGFß-1 plasma levels throughout the immunotherapy (see Figure 2).

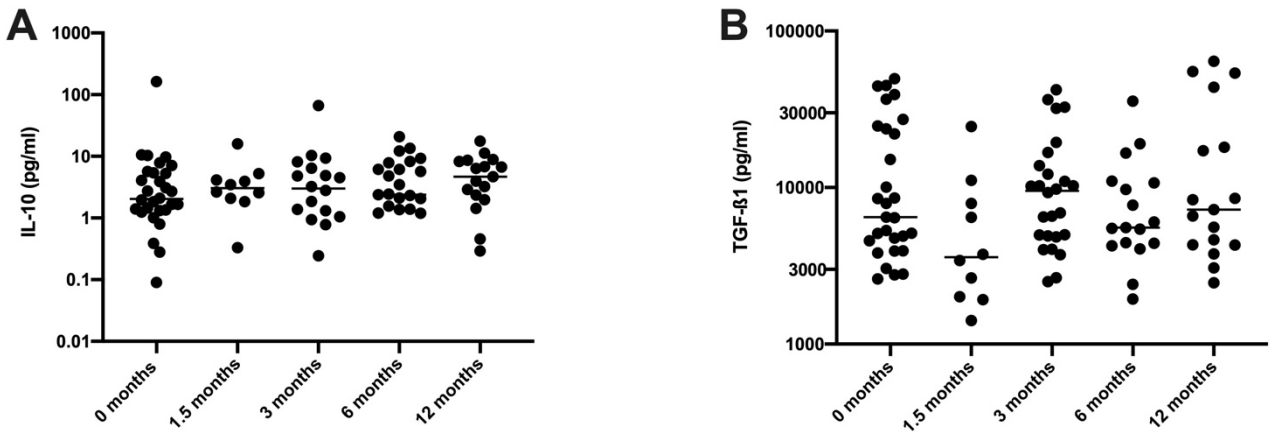

Figure 2. Concentrations of (A)IL-10 and (B) TGFß-1 during immunotherapy (on a logarithmic scale). The horizontal line indicates the median.

Analyzing the groups separately, there was a significant difference (p = 0.0326) when comparing the IL-10 concentrations at the initiation and end of the study in the ILIT group (see Figure 3A).

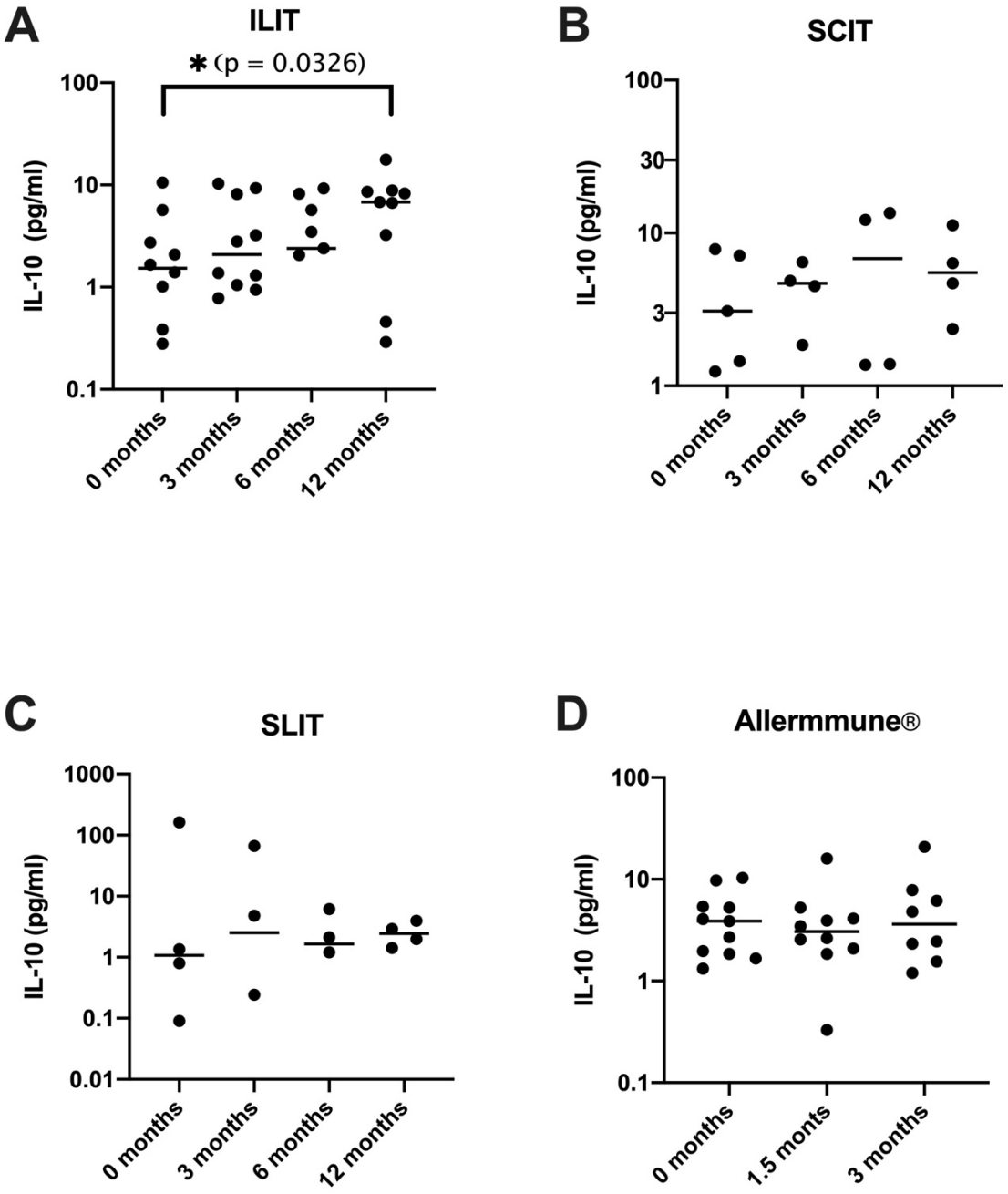

Figure 3. IL-10 concentrations during immunotherapy (on a logarithmic scale). (A) In the ILIT group the dogs showed a significant increase of IL-10 values between the initiation of the treatment and the completion of the study (p = 0.0326). (B) SCIT group. (C) SLIT group. (D) Allermmune® group. The horizontal line indicates the median.

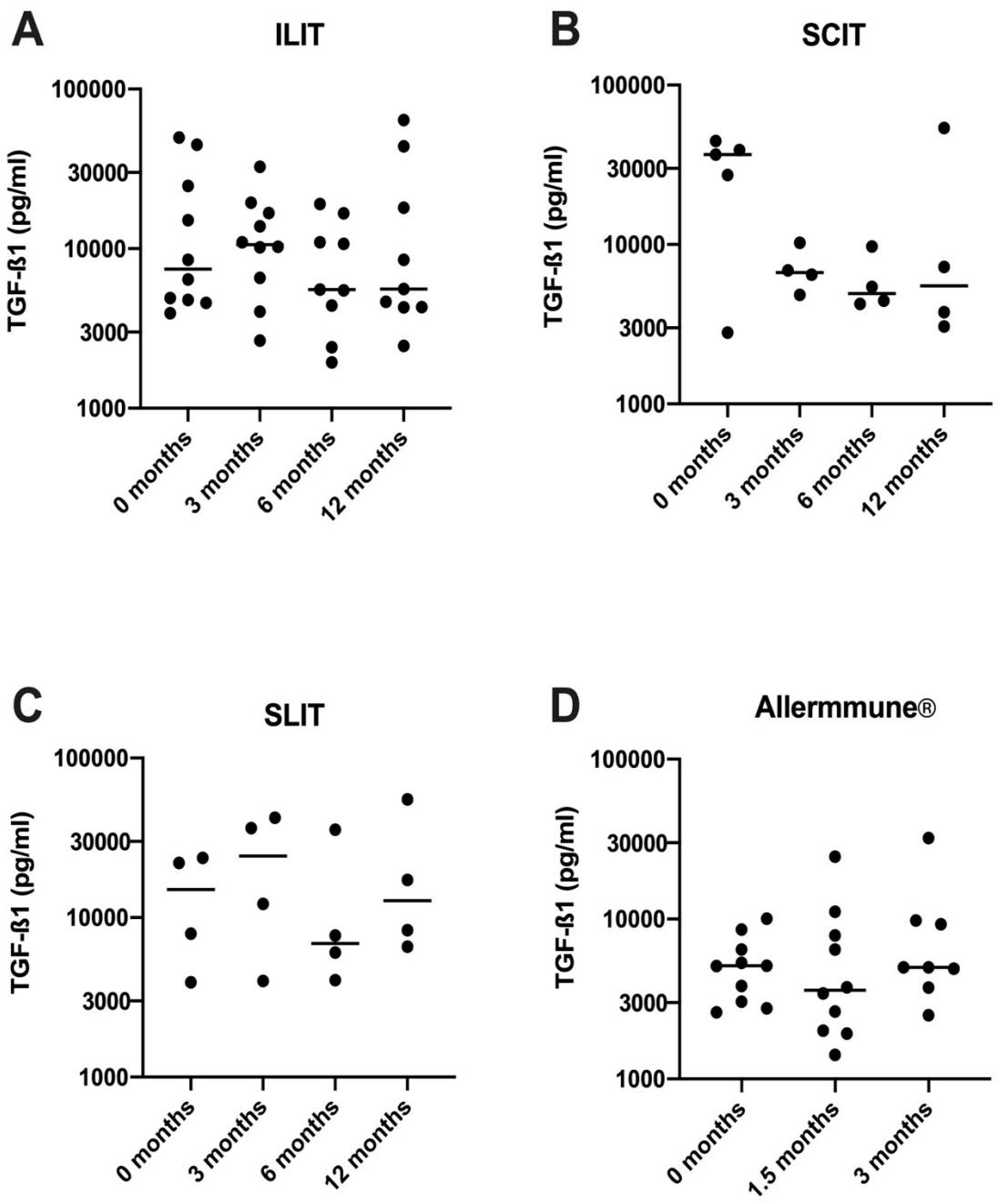

Figure 4. TGFß-1 concentrations during immunotherapy (on a logarithmic scale). (A) ILIT group, (B) SCIT group, (C) SLIT group. (D) Allermmune® group. The horizontal line indicates the median.

There was no statistical difference between dogs who clinically responded and non-responders. The IL-10 and TGFß-1 levels of the responders didn't show any significant alteration during the immunotherapy and cytokine levels of responders and non-responders at completion of the study were also not statistically different.

Discussion

There is very little known about the role regulatory cytokines and Tregs in the pathogenesis of canine atopic dermatitis. A recent report showed that allergic dogs had significantly higher serum TGFß-1, but lower IL-10 levels.[20] While other studies have reported significantly lower IL-10 and TGFß-1 values in allergic dogs,[21] or like in the present work no difference at all between the two groups.[16,23,24]
These controversial results may reflect the complex mechanisms underlying immune response regulation and IL-10 and TGFß-1 production in the canine body. It is known that the biological behavior of some cytokines can vary depending on the stage of the disease, concurrent medications and interplay between different mediatiors.[30] Some cytokines may also show both pro-inflammatory and regulatory activity. IL-10 for example, can either downregulate the immune response, or mediate a Th2-type inflammation.[31] Thus, it could be challenging to determine whether this cytokine is actively contributing to the inflammatory response or is trying to suppress it.
Differences in breed or age distribution in the study populations may also be reflected in the regulatory cytokines' levels. One study has recently reported a highly significant breed-influence on the circulating levels of TGFß-1 to support this idea.[32]
As mentioned above, concurrent medications can influence the biological behavior of some cytokines. In the present study, in contrast with other reports[16,20] atopic dogs were included in the study even if they were under concurrent treatment with corticosteroids or other immunosuppressive medications. However, analyses were also repeated after with the exclusion of those dogs and the removal did not modify the outcome.
In the present work no differences in the plasma levels of IL-10 and TGFß-1 between atopic and control dogs were observed. This may seem contradictory since most of the previous studies showed that atopic dogs have higher percentage of circulating Tregs than healthy dogs.[13,14,21] A possible explanation is that atopic dogs could have non-functional or non-activated Tregs that do not produce regulatory cytokines. It is also possible that markers used in previous studies to characterize canine Tregs were not adequate. In fact, several cell markers have been used to define Tregs in mice or humans and many of these are still not available for dogs.[19] Another hypothesis would be that IL-10 and TGFß-1 may not be the main regulatory cytokines in atopic dogs, and that their main regulatory factors still has yet to be discovered.

Recent human studies demonstrated that the generation of Tregs and increased production of regulatory cytokines are key events in the mechanism of action of ASIT. [10,11] They act by suppressing of the proliferation of T cells and modulating the production of antibodies from IgE class towards IgG4 and IgA. [11,12,33]
In the present study a significant increase in IL-10 was observed at completion of the study in the ILIT group. It should be noted that treatment in the ILIT group included aluminum hydroxide, which prolongs exposure of the allergens to the immune system. We hypothesized that this prolonged exposure may be a possible explanation for this result. Furthermore, IL-10 is synthesized by a wide range of cells including Tregs, like Th2 cells, B cells, monocytes and dendritic cells. Those cells are present in high amount in the lymph nodes. We can speculate that by directly injecting the allergens into a lymphoid organ they are more likely to contribute to the production of IL-10.[34] The observed increase in IL-10 in this particular group could therefore reflect the better clinical efficacy of the intralymphatic delivery route, as already shown in our previous study.[9]
No significant IL-10 changes were observed in the other groups. As shown in a human study, just a small amount of allergens injected subcutaneously reach the lymph node via lymphatic drainage.[35] It is possible that this amount is too small to cause a significant

increase in IL-10. The efficacy of the immune response in SLIT depends on the contact time between the allergen and the oral mucosa, and avoidance of swallowing for a period of time afterward.[36] This procedure is obviously problematic in canine patients. The lack of significant changes in cytokines' levels in the SLIT could thus be explained by this fact. An important issue associated with these findings is that values below detection limit were included in the statistical calculation. By repeating the statistics including only values above detection, no statistical significance was observed. Nevertheless, we decided to include all the values following the recommendations of the Analytical Methods Committee of the Royal Society of Chemistry.[29] We assumed that simply ignoring the values below detection would have led us to more misleading results.

Similarly as in human patients, Keppel *et al* reported a significant increase of Tregs and serum IL-10 in 53 atopic dogs undergoing subcutaneous immunotherapy.[16] Comparing our results with those of this publication, it appears that the IL-10 values that we obtained are much lower and that our data was spread over a much wider range (see Table 1 and Figure 1). As mentioned above, strong variations in the study populations may be responsible for these findings. The method of detection of the cytokines could also have had an impact on the results. The ELISA Kits used in the present work have already been employed in several previous publications,[20,32,37–39] and the reported means or medians of the concentrations vary strongly. Possible manufacturers' changes in the composition of the kit or its reagents could be responsible for these changes.

The most important limitation of our study is the high number of IL-10 values below detection limit. However, we decided that including those values in the calculation (rather than excluding or substituting them) would provide results coming closer to the real situation.[29]
Besides ELISA, the parallel use of other detection methods, such as qPCR or Flow Cytometry, would have provided us with more information. In general, detection tests with higher sensitivity are needed to get better insights into the actual circulating levels of regulatory cytokines in canine peripheral blood.
The low number of dogs included in each immunotherapy group (especially in the SCIT and SLIT) is another important issue. We cannot exclude that with a larger study population significant changes would have been observed also in other groups.
Based on the results of the present study, we can assume that the plasma regulatory cytokines IL-10 and TGF-ß1 do not seem to efficient markers of the atopic status, nor can be used to monitor the course of the disease. It is difficult to determine whether they are involved to some extent in the mechanisms of action of ASIT. An efficient use of these as objective biomarkers to assess the success of an immunotherapy appears unlikely.

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